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Improving Anticancer Activities of *Oplopanax horridus* Root Bark Extract by Removing Water-soluble Components

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Abstract

Oplopanax horridus is used as a folk medicine by natives in the Northern Pacific coast of North America. This experiment studied the anti-proliferative effects of the extract of *O. horridus* root bark and its fractions chromatographed from Dianion HP20 resin column with water, 30, 50, 70 and 100% ethanol on human breast cancer MCF-7 cells and non-small cell lung cancer (NSCLC) cells. The role of *O. horridus* in the cell cycle and apoptosis of MCF-7 cells was also investigated. The results showed that the 70% and 100% ethanol fractions demonstrated more potent anti-proliferative effects than the total extract on both cell lines. The anti-proliferative effects may result from the enrichment of active constituents detected by the HPLC. The IC_{50} of the total extract, 50, 70, and 100% ethanol fractions for anti-proliferation on MCF-7 cells were 248.4, 123.1, 44.0, and 31.5 μg/mL, respectively, and on NSCLC cells were 125.3, 271.1, 17.6, and 23.2 μg/mL, respectively. On the other hand, the water and 30% ethanol fractions significantly promoted cell proliferation on MCF-7 cells at concentrations > 100 μg/mL, suggesting that the hydrophilic fractions should be removed from the extract when used for cancer chemoprevention in order to achieve desirable activities. The effects of the total extract on cell cycle and apoptosis were similar to that of the 100% ethanol fraction because of the similarity of their chemical composition. At higher concentrations, the apoptotic effects of the 70% ethanol fraction are more significant. Data from this study suggested that the 70% and 100% ethanol fractions are active anti-proliferative fractions and that induction of apoptosis is the mechanism involved in the anti-proliferative effect observed.

Keywords

Oplopanax horridus; anti-proliferative effect; human breast cancer MCF-7 cells; non-small cell lung cancer (NSCLC) cells; apoptosis; cell cycle

INTRODUCTION

Ginsengs, which belong to the family Araliaceae, are a group of perennial aromatic herbs widely used in Oriental medicine and include Asian ginseng (*Panax ginseng*), North American ginseng (*P. quinquefolius*) and notoginseng (*P. notoginseng*) (Wang *et al.*, 2009; Wang and Yuan, 2008). *Oplopanax* (*Echinopanax*) is a small genus also belonging to the

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family Araliaceae. In this genus, there are only three species whose ranges are rather narrow. *O. elatus* grows in the southern regions of Primorye, northeastern China, and north of the Korean Peninsula; *O. japonicus* in Japan; and *O. horridus* (Devil's club) in the Northern Pacific coast of North America (Artyukova *et al.*, 2005; Xiang and Lowry, 2007).

The three species of *Oplopanax* have been used in folk medicine by natives of the region that originate from (Academy of Traditional Chinese Medicine and Traditional Chinese Herb of Ji-lin Province, 1982; Lantz *et al.*, 2004; Takeda *et al.*, 1966). One species, *O. horridus*, is even regarded to be on par with Oriental ginseng and is used as a body-balancing and system-strengthening tea (Schofield, 2000). In traditional medicinal use, the stem and root of *O. elatus* is used for treating neurasthenic, hypopiesis, schizophrenia, cardiovascular diseases, diabetes mellitus and rheumatism (Academy of Traditional Chinese Medicine and Traditional Chinese Herb of Ji-lin Province, 1982); the root, bark and stem of *O. japonicus* are used as an antipyretic or cough medicine (Takeda *et al.*, 1966); the bark or berries of *O. horridus* are often used in the form of an extract for the treatment of arthritis (inflammation), hyperglycemia, gastrointestinal disorders, infections and respiratory ailments (Johnson, 2006; Moore, 1993; Schofield, 2000). It is believed that the dammarane-type triterpenoids are related to the bioactivities of ginseng (Kang *et al.*, 2008; Zuo *et al.*, 2009). Based on previous observations, the anticancer components of processed ginseng are reliant on its derived ginsenosides (Wang *et al.*, 2007a; Wang *et al.*, 2007b; Yun *et al.*, 1983). *O. elatus*, on which the phytochemical and physiological studies are reported, is found to contain triterpenoids, specifically oleanane-type and lupene-type triterpenoids whose structures differ from that of dammarane-type ginsenosides (Wang, 2006). *O. horridus* is reported to contain sesquiterpenes which include equinopanacene, equinopanacol (Kariyone and Morotomi, 1927), ox-cubebene,, spathulenol, oplopanone (Bloxton and Marderosian, 2002), 3,10-epoxy-11-hydroxynerolid-6-ene, and 3*S*,6*R*,7*S*, 10*R*-*cis*-6, 11-dihydroxy-7, 10-epoxynerolidol (Inui, 2008); 5 polyynes includes falcarinol, falcarindiol, oplopandiol, 9,17-octadecadiene-12,14-diyne-1,11,16-triol, 1-acetate, and oplopandiol acetate (Kobaisy *et al.*, 1997); and one liginin, sesamin (Inui, 2008). In addition, lignan 1,3 benzodioxole, 5,5'-tetrahydro-1H,3H-furo[3,4-c]furan- 1,4-diyl)bis, stearic acid, stigmasterol and β-sitosterol were identified by comparison (Bloxton and Marderosian, 2002; Gruber *et al.*, 2004). Among them, polyynes were directly identified to display significant antimycobacterial, antimicrobial, and antifungal activity (Kobaisy *et al.*, 1997). However, other components of *Oplopanax* were also reported to be active. For example, trans-nerolidol with anti-colon cancer effects in rats (Wattenberg, 1991) and spasmolytic effect in mice (1966). Stigmasterol and β-sitosterol with antirheumatic and anticholesterolemic properties (1989). The bioactivities of *O. horridus* have been evaluated by pharmacological tests. For example, studies conducted by Large and Brocklesby exhibited marked hypoglycemic property attributable to the extract (Large and Brocklesby, 1938), though this effect can not be confirmed in a subsequent clinical study (Smith, 1983). Tests by McCutcheon have proven the extract to display antiviral effects against respiratory syncytial virus (McCutcheon *et al.*, 1995). McCutcheon (McCutcheon *et al.*, 1997) and Inhu (Inui, 2008; Inui *et al.*, 2007) evaluated the inhibitory effects of *O. horridus* bark and root extract on *Mycobacterium tuberculosis* cell growth *in vitro*. Tai (Tai *et al.*, 2006) reported the anti-proliferative effect of 70% ethanol extract of *O. horridus* root bark on several cancer cell lines, K562, HL60, MCF7, and MDA-MB-468.

The profile of *O. horridus* fractions responsible for the anti-cancer activities and their related mechanism, however, has not been investigated. This experiment was designed to study the anti-proliferative effects of the different fractions chromatographed from *O. horridus* root bark by Dianion HP20 resin on breast cancer MCF-7 cells and non-small cell lung cancer cells (NSCLC). In addition, the role played by different compositions in the MCF-7 cell cycle and apoptosis was also investigated.

EXPERIMENTAL DETAILS

Chemicals

All solvents were of high-performance liquid chromatography (HPLC) grade (Fisher Scientific, Norcross, GA). Milli Q water was supplied by a water purification system (US Filter, Palm Desert, CA). Plastic materials were purchased from Falcon Labware (Franklin Lakes, NJ). Trypsin, Leibovitz's L-15 medium, fetal bovine serum (FBS), and penicillin/streptomycin solution (200×) were obtained from Mediatech, Inc. (Herndon, VA). A CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit was obtained from Promega (Madison, WI). An Annexin V-FITC Apoptosis Detection kit was obtained from BD Biosciences (San Diego, CA). Other reagents were purchased from Fisher Scientific (Pittsburgh, PA).

Plant Material

The root bark of *Oplopanax horridus* (Sm.) Miq was purchased from Pacific Botanicals, Grants Pass, OR. A voucher specimen (no. 20080420-1) was deposited in the Tang Center for Herbal Medicine Research at the University of Chicago.

Extraction and Isolation

Dried and ground root bark of *O. horridus* (2.4 kg) was extracted with 70% ethanol (1: 8, W: V) refluxed at 90°C for 4 h and filtered, the residue was extracted with 70% ethanol (1: 6, W: V) refluxed at 90°C for 2 h two more times. The filtrates were combined and evaporated at 60°C *in vacuo* to acquire a total extract (503.2g, 20.97%). The ethanol extract was applied to Dianion HP-20 (Sorbent Technologies, Atlanta) column chromatography (75×610 mm, Ace Glass, Inc., Vineland, NJ) eluting with a H_2O-EtOH gradient system (0, 30, 50, 70,100% ethanol) to give corresponding fractions of 284.0, 38.5, 56.0, 75.0 and 53.5 g, respectively. The experiment was performed twice. Thin layer chromatography (TLC) was carried out on TLC Silica gel 60 F_{254} plates and TLC RP-18 F254 (EMD Chemicals Inc., Darmstadt, Germany), and spots were visualized by UVLMS38 (UVP, LLC., Upland, CA) and by spraying the plates with 10% H_2SO_4 of ethanol solution followed by heating.

Sample Preparation

For HPLC samples, weighted 40 mg samples were dissolved with MeOH/H2O into 10 mg/mL concentrated solutions (70% fraction into 5mg/mL), the solutions were then filtered with Millex 0.2 μm nylon membrane syringe filters (Millipore Co., Bedford, MA) to vials for HPLC analysis. For bioassay samples, weighted 30 mg samples were dissolved with DMSO or 70% ethanol into 30 mg/mL solutions, the solutions were diluted into 300, 100, 30, 10, 3, 1, 0.3 and 0.1 μg/mL for the bioassay. Stock solutions were stored at −20°C before use.

HPLC Instrumentation and Analysis

The HPLC system included a Waters 2965 instrument (Milford, MA) with a quaternary pump, an automatic injector, a photodiode array detector (Model 996), and Waters Millennium 32 software for peak identification and integration. The separation was carried out on a 250×3.2 mm i.d., 5 μ, Prodigy ODS (2) column (Phenomenex, Torrance, CA) with a 7.5× 3.2 mm i.d. guard column. For HPLC analysis, a 20 μL sample was injected into the column and eluted at room temperature with a constant flow rate of 1.0 mL/min. For the mobile phase, acetonitrile (solvent A) and water (solvent B) were used. Gradient elution started with 30% solvent A and 70% solvent B, and held for 1 min. The elution was changed to 36% A for 9 min, to 50% A for 9 min, to 68% A for 10 min, to 80% A for 3 min, to 90% A for 4 min and held for 3 min. The last elution was changed to 30% A for 5 min and held

for 7 min. The detection wavelength was set to 202 nm, and the wavelength range was 196–450 nm.

Cell Culture

The human breast cancer cell lines MCF-7 (RPMI-1640) and non-small cell lung cancer cells (NSCLC, DMEM) were purchased from American Type Culture Collection, ATCC, (Manassas, VA) and grown in the indicated media supplemented with 10 % FBS and 50 IU penicillin/streptomycin in a humidified atmosphere of 5 % CO_2 at 37 °C.

Cell Proliferation Analysis

Cells were seeded in a flat-bottomed 96-well plate with a multichannel pipet (1×10^4 cells/well). After 24 h, the medium was removed and 200 μL of fresh culture medium was added to each well. Various concentrations of extract and fractions were added to the wells. The final concentration of DMSO or ethanol tested groups was 0.1or 0.5%. Controls were exposed to culture medium containing the same quantity of DMSO or ethanol without drugs. All experiments were performed at least three times. At the end of the drug exposure period (48 h), the medium was removed from all the wells and 100 μL of fresh medium plus 20 μL of CellTiter 96 aqueous solution was added to each well. CellTiter 96 aqueous solution is composed of a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, an electron-coupling reagent (phenazine methosulfate), and buffer. When the solution contacts viable cells, it is bioreduced by dehydrogenase enzymes in metabolically active cells into a formazan product. The quantity of formazan product, measured by the amount of absorbance at 490 nm, is directly proportional to the number of living cells in culture. The plate was then incubated for 1 h in a humidified atmosphere at 37°C. 60 μL of medium from each well was transferred to an ELISA 96-well plate, and the absorbance of the formazan product at 490 nm was measured. The blank was recorded by measuring the absorbance at 490 nm with wells containing medium but no cells. All observations were performed in triplicate. Results were expressed as percent of control (solvent vehicle set at 100%).

Cell Cycle Assay

The MCF-7 cells were seeded in 24-well tissue culture plates. On day 2, the medium was changed and the cells were treated with extract/fractions. The cells were incubated for 48 h before being harvested. The cells were fixed gently by adding 80% ethanol and placing them in a −20 °C freezer for 2 h. They were then treated with 0.25% Triton X-100 for 5 min in an ice bath. The cells were resuspended in 300 μL of PBS containing 40 μg/mL propidium iodide and 0.1 mg/mL RNase. Then the cells were incubated in a dark room for 20 min at room temperature, and analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and FlowJo 7.1.0 software (Tree Star, Ashland, OR). For each measurement, at least 20,000 cells were counted.

Apoptosis Assay

The MCF-7 cells were seeded in 24-well tissue culture plates. After culturing for 1 day, the medium was changed and the extract/fractions were added. After treatment for 48 h, the cells floating in the medium were collected. The adherent cells were detached with 0.05% trypsin. Then the culture medium containing 10% FBS (and floating cells) was added to inactivate the trypsin. After being pipetted gently, the cells were centrifuged for 5 min at 1500 g. The supernatant was removed and the cells were stained with annexin V-FITC and PI according to the manufacturer's instructions. Annexin V-FITC detects translocation of phosphatidylinositol from the inner to the outer cell membrane during early apoptosis, and PI can enter the cell in late apoptosis or necrosis. Untreated cells were used as control for the

double staining. The cells were analyzed immediately after staining using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and FlowJo 7.1.0 software (Tree Star, Ashland, OR). For each measurement, at least 20,000 cells were counted.

Statistical Analysis

Data are presented as mean ± standard error (SE). A one-way ANOVA determined whether the results had statistical significance. In some cases, Student's *t*-test was used for comparing two groups. The level of statistical significance was set at $P < 0.05$.

RESULTS

Column chromatography and HPLC profile

EtOH-H_2O as the gradient elute was 0, 30, 50, 70, and 100% ethanol. HPLC chromatograms (Figure 1A–F) showed that 4 major and 9 minor components detectable in the root bark extract of *O. horridus* may be effectively enriched by the column chromatography of Dianion HP-20 resin. For 100% ethanol elute, the total number of peaks detectable under UV 202 nm was 34. These peaks not only include all components detected in the total extract, but also other components which were not observed in the extract. The 70% elute had a larger proportion of 4 major components as compared to the other fractions; There were hardly any detectable components in the water and 30% fraction in HPLC chromatogram under UV 197–450 nm. The relative content of the components in the 70% and 100% ethanol fractions increased significantly, 10.64 and 7.42 folds respectively, by the area normalization method (Figure 2). There was greater accumulation of 4 major components in the 70% fraction (5.10 folds) than in the 100% fraction (1.98 folds).

Anti-proliferative activity

Figure 3 showed the anti-proliferative activities of the chromatographic fractions of the extract of *O. horridus* root bark subjected to macroporous resin on human breast cancer MCF-7 cells and non-small cell lung cancer (NSCLC) cells. The results showed that the hydrophobic fractions were more active than the hydrophilic fractions: The 70% and 100% fractions were the most active fractions. The IC_{50} of the total extract and 50%, 70%, and 100% fractions of anti-proliferation on MCF-7 was at 248.4, 123.1, 44.0, and 31.5 μg/mL respectively. On NSCLC cells, the IC_{50} was 125.3, 271.1, 17.6, and 23.2 μg/mL respectively. However, water and 30% ethanol fractions promoted MCF-7 cell proliferation at concentrations > 100 μg/mL.

Effects of extracts on MCF-7 cell cycle

To determine whether the inhibitive mechanism of the total extract and fractions on MCF-7 cells proliferation involved cell cycle changes, cell cycle phases distribution of the treated cells were examined by flow cytometry. As shown in Figure 4, when the cells were treated with 30 μg/mL of the total extract and fractions, the total extract increased the percentage of cells in the G_1-phase to 49% and decreased S-phase to 6.22%. The 70% ethanol fraction decreased the percentage of cells in the G_1-phase to 34.4%. The 100% ethanol fraction decreased the percentage of cells in the S-phase to 6.22%. When the cells were treated with 100 μg/mL samples, the total extract increased the percentage of cells in the G_1-phase to 47.2% and G_2-phase to 22.5%. For the 70% and 100% ethanol fractions, cell cycle profiles could not be detected because there were not enough viable cells to be collected. The 30% ethanol fraction, contrasted to the control, had hardly any influence on the cell cycle.

Apoptotic effect of extracts on MCF-7 cells

As shown in Figure 5, at a concentration of 30 μg/mL, only the total extract showed marked inductive effect on the apoptosis of MCF-7 cells, early apoptosis was 29.9%, late apoptosis/necrosis 15.5% after treatment for 48 h. At a concentration of 100 μg/mL, compared to the total extract (early apoptosis 18.1%, late apoptosis/necrosis 12.9%) and 30% ethanol fraction (early apoptosis 7.64%, late apoptosis/necrosis 13.5%), the 70% and 100% ethanol fractions significantly increased early apoptosis to 49.1% and 40.9%, and late apoptosis/necrosis to 39.5% and 30.2%, respectively. For the viable cells, the control was 77.3%, total extract and 30% fraction at 100 μg/mL concentration were 68.4% and 77.9%, respectively, while the 70% and 100% fractions were 11.3% and 27.8%, respectively. At a concentration of 300 μg/mL, the 30% ethanol fraction had no effect on MCF-7 cells (early apoptosis 8.15%, late apoptosis/necrosis 14.4%, and viable cell 76.9%). The total extract significantly increased apoptosis (early 45.6%, late 18.8%, viable cell 34.4%) and the 70% and 100% ethanol fractions markedly reduced the proportion of the viable cells.

DISCUSSION

Herbal extracts are comprised of a complicated mixture of secondary metabolites which may possess biological activities. The active components can be enriched by partitioning or chromatographic methods. In this experiment, the active components, which should be hydrophobic, were effectively enriched in 70% and 100% ethanol fractions chromatographed from Dianion HP20 column (Figures 1 and **2**). From the root bark of *O. horridus*, Kobaisy et al (1997) showed its major components were polyacetylenes, and this observation supported by current study using HPLC-UV determination.

Previous studies demonstrated anti-proliferation effects of polyacetylenes from plants of families Araliaceae and Apiaceae (Christensen and Brandt, 2006). Naturrally occurring polyacetylenes possess cytotoxic effects on different cancer cell lines (Dembitsky, 2006), such as mouse malignant melanoma cells (B16) and fibroblast derived tumor cells (L-929), human gastric carcinoma MK-1 cells, breast carcinoma M25-SF and MCF7 cells ovarian cancer cell lines SK-OV-3, and hepatocarcinoma cell line HepG2 (Guo *et al.*, 2009).

We assayed anti-proliferative effect, which also called cytotoxic effect in literature, of different fractions of *O. horridus* on human breast cancer MCF-7 cells or NSCLC cells using MTS method, and observed significant pharmacological activities in a concentration-dependent manner.

In MCF-7 cells, either proliferative or anti-proliferative effects may occur in different *O. horridus* fractions at different concentrations. The small or non-polar (70% and 100% ethanol) fractions significantly increased anti-proliferation, while the polar (water and 30% ethanol) fractions promoted proliferation of MCF-7 cell at over 100 μg/mL (Figure 3A). This result suggested that promoting proliferative components and anti-proliferative components may coexist in the total extract and interact to influence the total extract's effect on the cancer cells. To improve efficiency, the two hydrophilic fractions should be removed from the root bark extract if it is to be used in the treatment of certain cancers.

Functional mechanisms involved in the anti-proliferative effects of *O. horridus* extract and fractions were evaluated. Cancer is frequently considered to be a disease of disruption of equilibrium by loss of cell cycle control (Sandal, 2002). Some compounds, inhibiting cell cycle progression, were developed as anti-tumor agents (Welburn and Endicott, 2005). In this study, we observed the total extract increase the percentage of cells in G2/M-phase, and reduce the cell percentage in S-phase. For the active fractions, 70% fraction reduced the percentage of cells in G1-phase, while 100% fraction reduced cells in S-phase (Figure 4).

Since diverse cell cycle profile changes were observed among extract and active fractions, cell cycle arrest may not play the key role in the cancer cell inhibition of the extract and fractions.

Apoptosis is programmed cell death, a highly regulated process used to eliminate unwanted or defective cells (Pucci *et al.*, 2000). Many chemotherapeutic agents and natural compounds, radiation, immunotherapy, and cytokines induce cancer cell death via the apoptotic pathway (Yun *et al.*, 1983; Lowe and Lin, 2000). We observed *O. horridus* extracts and fractions on the apoptotic induction of MCF-7 cells. The total extract and the two active fractions obviously induced cell apoptosis. The 70% fraction, which possessed most potent anti-proliferative activity, showed the strongest apoptotic induction activity (Figure 5). This result suggested that the anti-proliferative effects of *O. horridus* extracts and active fractions were mediated by the induction of apoptosis.

Data from this study showed that the root bark extract of *O. horridus* and 70% and 100% ethanol fractions chromatographed from Dianion HP20 resin column presented significant inhibitory effects on human breast cancer MCF-7 cells and non-small cell lung cancer (NSCLC) cells. The anti-proliferative activities increased with the accumulation of the active secondary metabolites in the non-polar fractions. Since the water and 30% ethanol fractions promoted cell growth in the MCF-7 cells at certain concentrations, these fractions should be removed from the extract to ensure desirable chemopreventive activities. The responsible constituents and the detailed functional mechanisms remain to be further investigated.

Acknowledgments

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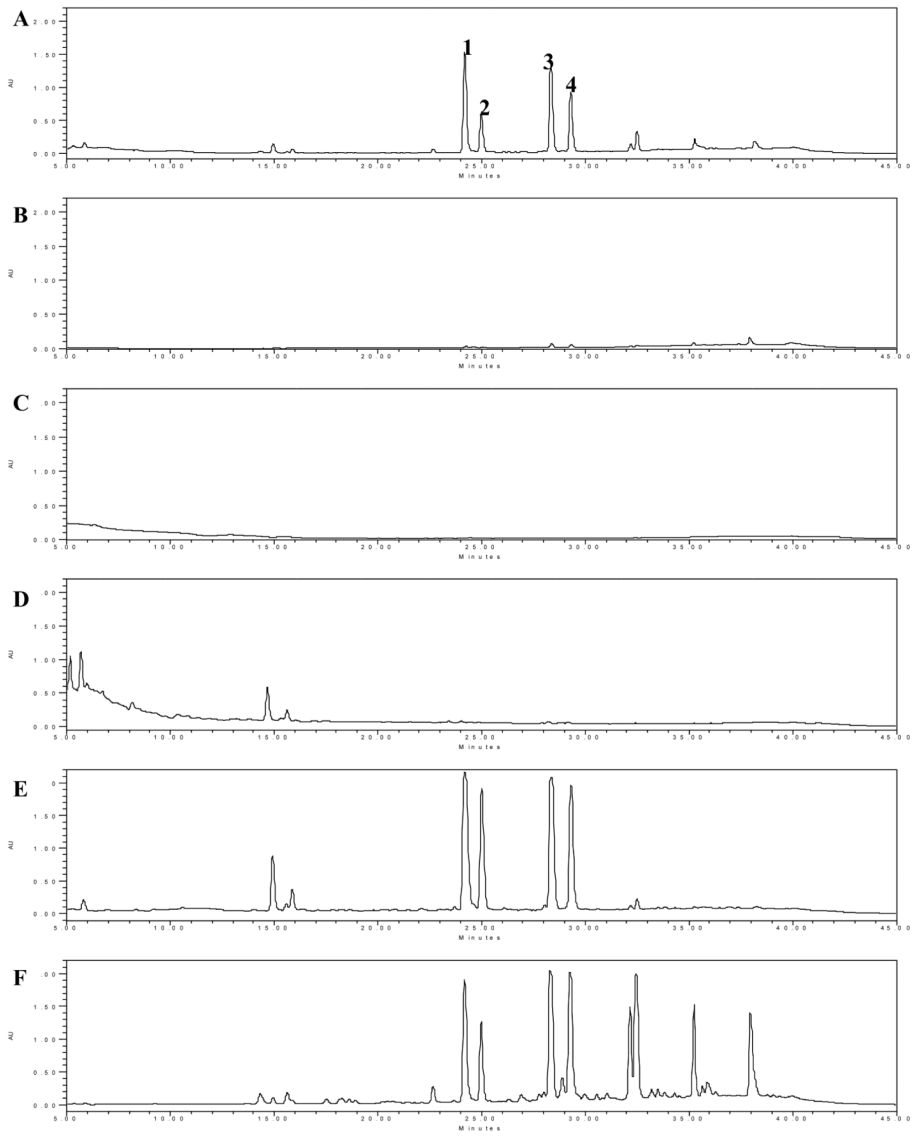

Figure 1.

HPLC-UV Chromatograms of the total extract from the root bark of *O. horridus* and fractions chromatographed from Dianion HP20 resin column at UV λ=202 nm. **A:** total extract (10mg/mL); **B–F:** 0% (10mg/mL), 30% (10mg/mL), 50% (10mg/mL), 70% (5mg/mL), and 100% (10mg/mL) ethanol eluted fractions from Diaion HP-20 column.

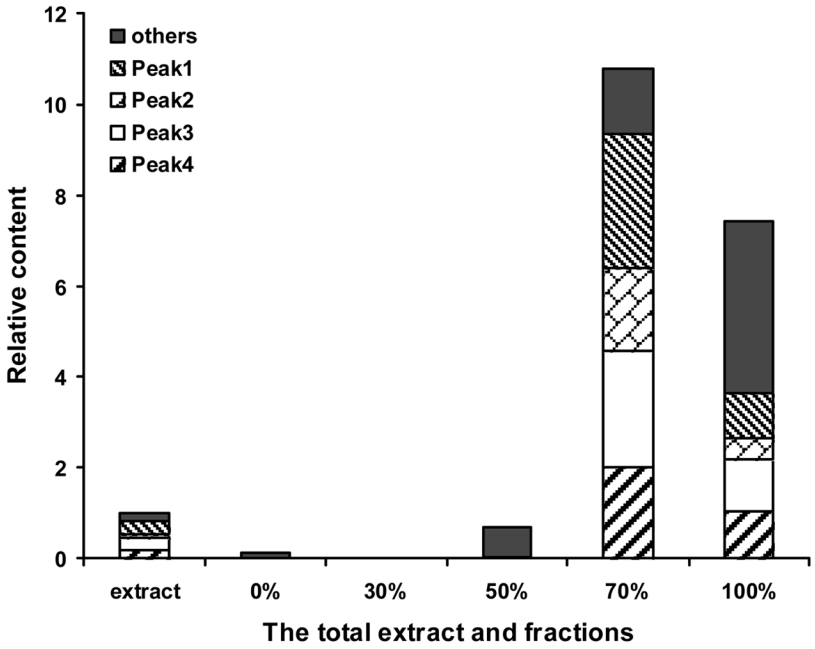

Figure 2.
The relative content of components from the root bark of *O. horridus* detectable in HPLC at UV λ=202 nm. The relative content values are the ratio of each peak area to the total peak areas of the total extract.

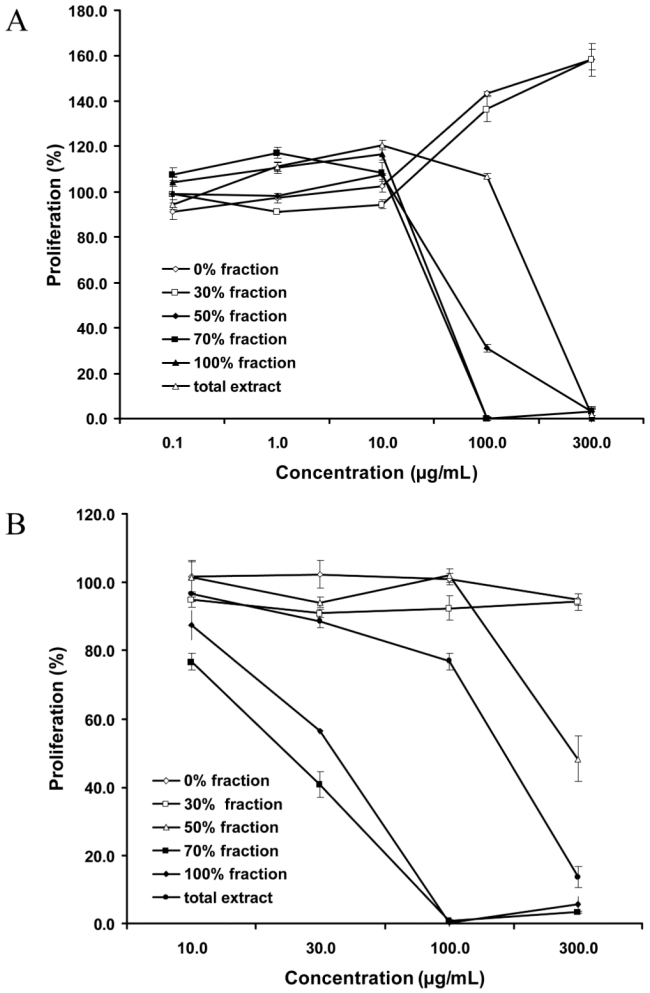

Figure 3.

Percentage of proliferation of human (**A**) breast cancer MCF-7 cells and (**B**) non-small cell lung cancer (NSCLC) cells treated for 48 h with the root bark extract of *O. horridus* and the fractions.

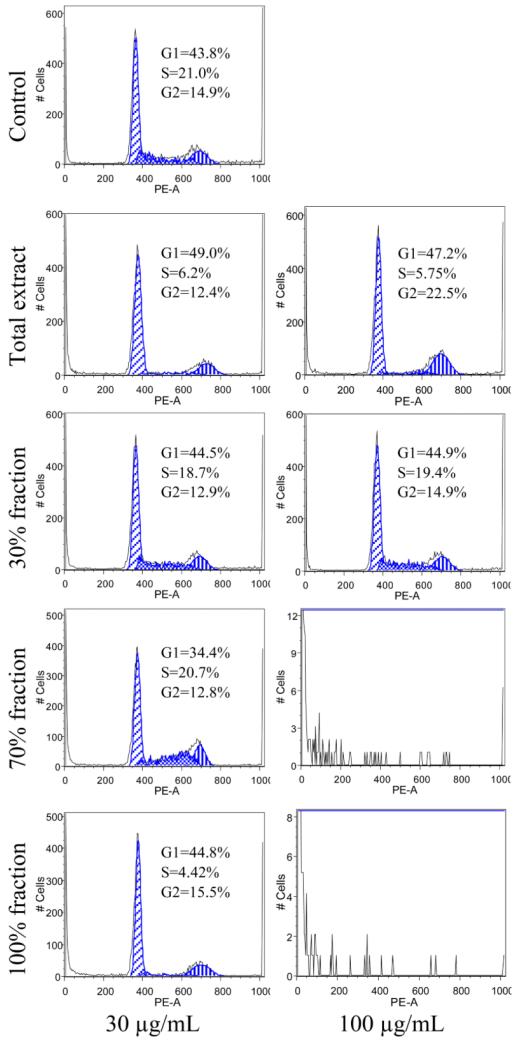

Figure 4.

Effects of root bark extract of *O. horridus* and the fractions on MCF-7 cell cycle. After treatment with the total extract or fractions for 48 h, the cells were stained with PI and assayed using flow cytometry. The percentage of cells in G_1-, S- and G_2/M-phases are indicated.

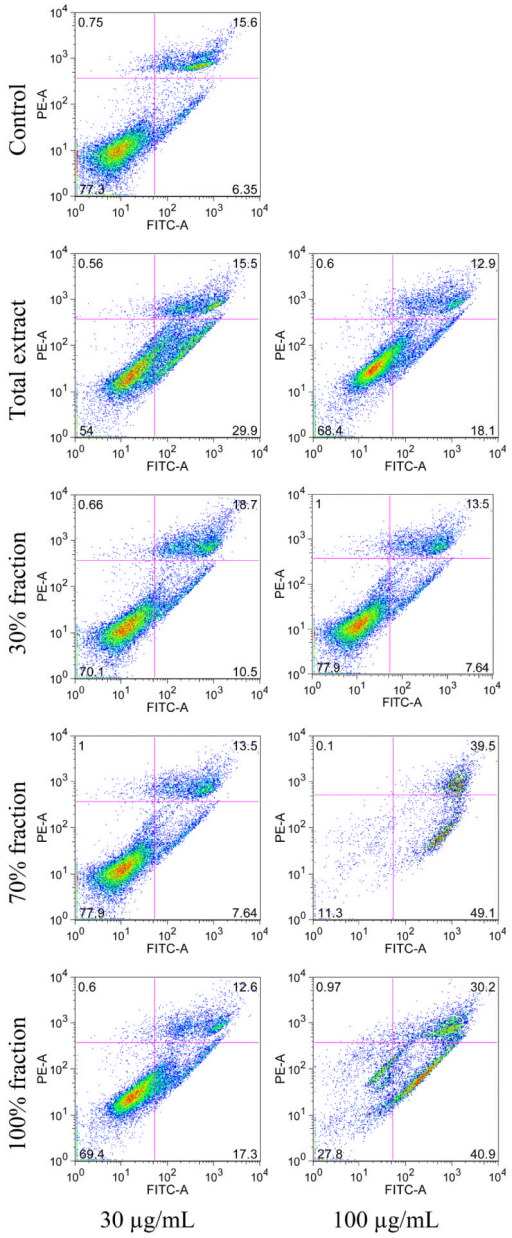

Figure 5.

Apoptosis assay using flow cytometry after annexin V-FITC/propidium iodide (PI) staining. MCF-7 cells were treated for 48 h with *O. horridus* extract and fractions. Viable cells are in the lower left quadrant, early apoptotic cells are in the lower right quadrant, late apoptotic or necrotic cells are in the upper right quadrant and non-viable necrotic cells are in upper left quadrant.